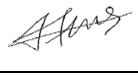
STANDARD OPERATING PROCEDURE (SOP)

SOP Title:	**IN-HOUSE CAGE (WEB VERSION)**
SOP Number:	SOP006-02
Effective Date:	27 APR 2020
Current Review Date:	27 APR 2022
Replaces SOP Number:	SOP006-01
Group:	GIH

I have read this document and approve its contents.

	Name	Team	Signature	Date
Written By:	Sohye Yoon	GIH single cell		27 APR 2020
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SOP006-01	Sohye Yoon	30 JUL 2019
SOP006-02	Sohye Yoon/Stacey Andersen	27 APR 2020

A. PURPOSE AND APPLICATION

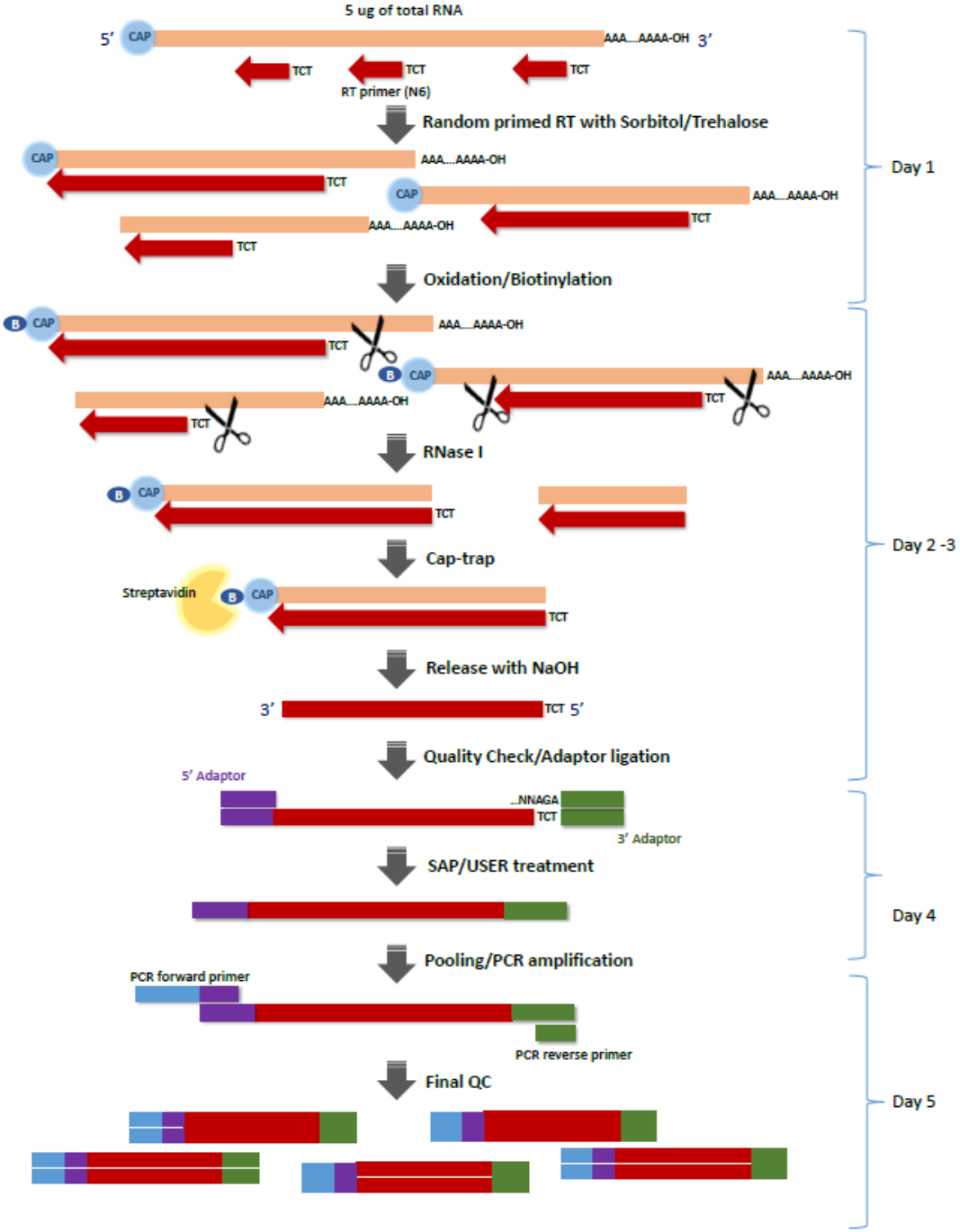
This SOP describes the generation of CAGE libraries via the cap-trapping method using an optimized protocol designed and validated in-house. This in-house protocol is based on the commercially-available kit from DNAFORM, but is shorter and includes a final PCR step to generate higher concentration libraries. Resulting libraries are full-length (un-fragmented), Illumina-compatible libraries containing both polyA and non-polyA transcripts, that have been minimally amplified by PCR. The entire process requires 5ug RNA/sample with RIN >7.

B. BRIEF SUMMARY OF METHOD

RNA is reverse-transcribed to generate an RNA/DNA hybrid. The methylguanylate cap on the 5' end of capped transcript is oxidised and biotinylated, and the duplex digested with RNAse I to release and remove the biotinylated 3' ends of RNA molecules. The biotinylated capped ends are captured with streptavidin beads, and 5' end cDNAs are released from the beads with NaOH. 5' and 3' linkers are added through two rounds of linker ligation, followed by USER/SAP treatment to manage unreacted adaptors. Samples are pooled before low-cycle PCR to generate completed library ready for sequencing.

Quality control checks are performed after cap-trapping and RNA digestion, after linker ligation prior to pooling, and after PCR prior to sequencing.

See below for a diagram outlining the procedure:



C. DEFINITIONS AND ABBREVIATIONS

CAGE – Cap Analysis Gene Expression, a method of generating sequence data for 5' ends of transcripts

D. OCCUPATIONAL HEALTH AND SAFETY

Users must read, understand, and sign on to Risk Assessment ID #2591 "Cap-analysis gene expression (CAGE) with CAGE Library preparation kit and in-house protocol" in the IMB Risk Management database. A pdf version of this risk assessment is available on the CAGE development page of the GIH website.

E. CAUTIONS

RNA is quickly degraded by RNAses present in the environment and the sample. Create an RNase free environment prior to starting work by destroying RNase on equipment (pipettes, ice buckets, tube racks, etc.), benchtops and gloves by wiping with RNase Decontamination Solution, such as RNaseZap®, and wearing lab coats and safety goggles. Treat equipment and gloves during the experiment as well in case they come into contact with un-treated equipment and areas. RNA samples must be placed on ice anytime unless there is specific instruction. It must be kept at -80°C for longer storage.

The protocol is optimized to prepare CAGE libraries using 5 µg/sample of total RNA. Insufficient quantity of total RNA may not be able to provide sufficient sample for sequencing. Meeting the following criteria is strongly recommended for the quality of total RNA to be used for the CAGE library preparation. Total RNA with low quality may not be able to provide sufficient sample for sequencing.

- A260/A230 ratio of ≧1.8 and A260/A280 ratio of ≧1.8 measured by spectrophotometer.
- RNA integrity number (RIN) of ≧7 measured by Agilent Bio-analyzer or equivalent.

All reagents should be prepared using RNA/DNA-free solutions and clean, dedicated equipment.

Reagents used for the oxidation and biotinylation are sensitive to light. Protect the reagents and samples from light as much as possible during these steps.

The cap-trapping protocol is particularly sensitive to residual ethanol in the reaction. Be sure to follow the extended drying steps at the end of the bead clean-ups to minimise ethanol carryover into the cap-trapping reactions.

F. PERSONNEL QUALIFICATIONS, TRAINING AND RESPONSIBILITIES

Training Requirements:

[X] Read and Understand Document [X] Training Required

G. EQUIPMENT AND MATERIALS

Equipment

a. Pipettes – P10, P20, P200, P1000. For many samples, multichannels may be useful.
b. SpeedVac vacuum concentrator
c. Benchtop centrifuge for 1.5mL tubes
d. Benchtop centrifuge for 8-strip tubes
e. Centrifuge for 96 well plates (for >8 samples)
f. Thermocycler

g. qPCR machine for 96 well plate (384 well plate if >8 samples)
h. Magnetic separator for 8-strip tube (96 well plate if >8 samples)
i. Agilent 2100 BioAnalyzer

Materials

a. Pipette tips – P10, P20, P200, P1000, plus multichannel tips as needed
b. 1.5mL LoBind tubes
c. 8-strip PCR tubes
d. Nuclease-free 96 well PCR plates
e. Nuclease-free 384 well PCR plates (for >8 samples)
f. OPTIPLATE-96 F /50B (Perkin Elmer; 6005270)
g. Optically-clear plate seals
h. Clean reagent reservoir (if using multichannel for washes)
i. Absolute ethanol
j. Isopropanol
k. Wet ice
l. RNAse Zap/ RNAseAWAY
m. Superscript III Reverse Transcriptase (ThermoFisher; 18080044)
n. RNACleanXP magnetic beads (Beckman-Coulter; A63987)
o. AMPureXP magnetic beads (Beckman-Coulter; A63881)
p. UltraPure water (ThermoFisher; 10977015)
q. Dynabeads M-270 Streptavidin (ThermoFisher; 65306)
r. Trehalose dihydrate (molecular biology grade; Life Sciences Advanced Technologies; TDH033)
s. d-Sorbitol (Sigma-Aldrich; 85529-250G)
t. NaIO4 (Sigma-Aldrich; 71859-25G)
u. Biotin (long arm) hydrazide (Vector Lab; SP-1100)
v. E. coli tRNA (ribonucleic acid, transfer from Escherichia coli Type XX, Strain W, lyophilized powder; Sigma;)
w. RQ1 RNase-free DNase (Promega; M6101)
x. Proteinase K (Invitrogen; 25530-049)
y. Trizol LS (Invitrogen; 10296-010)
z. Chloroform (Sigma-Aldrich; C2432)
aa. RNase ONE ribonuclease (Promega; M4261)
bb. Quant-iT OliGreen ssDNA Assay Kit (ThermoFisher; O11492)
cc. Agilent High Sensitivity DNA Kit (Agilent; 5067-4626)
dd. KAPA Library Quantification Kit for Illumina (KAPA Biosystems; KK4835)
ee. SYBR Green qPCR mastermix (ABI; 4312704)
ff. DNA ligation kit (Mighty Mix) (Takara; 6023)
gg. Phusion high-fidelity DNA polymerase (Finnzymes; F-530S)
hh. Exonuclease I (E. coli; NEB; M0293S)
ii. MinElute PCR purification kit (Qiagen; 28004)
jj. dNTPs (10 mM; Invitrogen; 18427-088)
kk. Sodium acetate (Sigma-Aldrich; S7899-100mL)
ll. EDTA (Thermo Fisher; AM9260G)
mm. Glycerol (Sigma-Aldrich; G5516-100ML)

nn. Tris-HCl (Astral scientific; BIOSD8141-450ML)
oo. Tris (1M) (ThermoFisher; AM9850G)
pp. NaOH (Sigma-Aldrich; 72068-100ML)
qq. MgCl2 (ThermoFisher; AM9530G)
rr. NaCl (ThermoFisher; AM9759)
ss. Sodium dodecyl sulfate solution 10% (Sigma-Aldrich; 71736-100ML)
tt. Wash buffers for MPG beads (wash buffers 1–4; see REAGENT SETUP)

Oligos

Name	Sequence	Purification Grade	Notes
RT primer	/5Phos/TCTNNNNNN	desalted	resuspend at 1mM
Hs_ACTB_qPCR_F	GGCATGGGTCAGAAGGATT	desalted	*for human samples
Hs_ACTB_qPCR_R	AGGTGTGGTGCCAGATTTTC	desalted	*for human samples
Hs_rRNA-qPCR_F	CTGGTTGATCCTGCCAGTAG	desalted	*for human samples
Hs_rRNA-qPCR_R	TCTAGAGTCACCAAAGCCGC	desalted	*for human samples
5'Ad1-upper	TTCCCTACACGACGCTCTTCCGATCT**ATCACG**NNNNNN/3Phos/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad1-lower	/5Phos/**CGTGAT**AGATCGGAAGAGCGTCGTGTAGGGAA/3AmMO/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad2-upper	TTCCCTACACGACGCTCTTCCGATCT**CGATGT**NNNNNN/3Phos/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad2-lower	/5Phos/**ACATCG**AGATCGGAAGAGCGTCGTGTAGGGAA/3AmMO/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad3-upper	TTCCCTACACGACGCTCTTCCGATCT**TTAGGC**NNNNNN/3Phos/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad3-lower	/5Phos/**GCCTAA**AGATCGGAAGAGCGTCGTGTAGGGAA/3AmMO/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad4-upper	TTCCCTACACGACGCTCTTCCGATCT**TGACCA**NNNNNN/3Phos/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad4-lower	/5Phos/**TGGTCA**AGATCGGAAGAGCGTCGTGTAGGGAA/3AmMO/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad5-upper	TTCCCTACACGACGCTCTTCCGATCT**TACAGTG**NNNNNN/3Phos/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)

5'Ad5-lower	/5Phos/**CACTGT**AGATCGGAAGAGCGTCGTGTAGGGAA/3AmMO/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad6-upper	TTCCCTACACGACGCTCTTCCGATCT**GCCAAT**NNNNNN/3Phos/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad6-lower	/5Phos/**ATTGGC**AGATCGGAAGAGCGTCGTGTAGGGAA/3AmMO/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad7-upper	TTCCCTACACGACGCTCTTCCGATCT**CAGATC**NNNNNN/3Phos/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad7-lower	/5Phos/**GATCTG**AGATCGGAAGAGCGTCGTGTAGGGAA/3AmMO/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad8-upper	TTCCCTACACGACGCTCTTCCGATCT**ACTTGA**NNNNNN/3Phos/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
5'Ad8-lower	/5Phos/**TCAAGT**AGATCGGAAGAGCGTCGTGTAGGGAA/3AmMO/	TruGrade	hand-mixed bases (N1:5,5,85,5)(N2:25,25,25,25)(N2)(N2)(N2)(N2)
3'Ad1-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**ATCACG**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad2-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**CGATGT**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad3-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**TTAGGC**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad4-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**TGACCA**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad5-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**ACAGTG**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad6-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**GCCAAT**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad7-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**CAGATC**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad8-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**ACTTGA**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad9-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**GATCAG**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	

3'Ad10-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**TAGCTT**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad11-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**GGCTAC**ATCUCGTATGCCGUCTTCTGCTTG	TruGrade	
3'Ad12-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**CTTGTA**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad13-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**AGTCAA**ATCUCGTATGCCGUCTTCTGCTTG	TruGrade	
3'Ad14-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**AGTTCC**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad15-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**ATGTCA**ATCUCGTATGCCGUCTTCTGCTTG	TruGrade	
3'Ad16-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**CCGTCC**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad17-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**GTAGAG**ATCUCGTATGCCGUCTTCTGCTTG	TruGrade	
3'Ad18-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**GTCCGC**ATCUCGTATGCCGUCTTCTGCTTG	TruGrade	
3'Ad19-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**GTGAAA**ATCUCGTATGCCGUCTTCTGCTTG	TruGrade	
3'Ad20-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**GTGGCC**ATCUCGTATGCCGUCTTCTGCTTG	TruGrade	
3'Ad21-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**GTTTCG**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad22-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**CGTACG**ATCUCGTATGCCGUCTTCTGCTTG	TruGrade	
3'Ad23-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**GAGTGG**ATCUCGTATGCCGUCTTCTGCTTG	TruGrade	
3'Ad24-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**GGTAGC**ATCUCGTATGCCGUCTTCTGCTTG	TruGrade	

3'Ad25-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**ACTGAT**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad26-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**ATGAGC**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad27-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**ATTCCT**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad28-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**CAAAAG**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad29-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**CAACTA**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad30-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**CACCGG**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad31-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**CACGAT**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad32-upper	NNNAGAUCGGAAGAGCACACGUCTGAACTCCAGUCAC**CACTCA**ATCUCGTATGCCGUCTTCUGCTTG	TruGrade	
3'Ad1-lower	CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad2-lower	CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad3-lower	CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad4-lower	CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad5-lower	CAAGCAGAAGACGGCATACGAGAT**CACTGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad6-lower	CAAGCAGAAGACGGCATACGAGAT**ATTGGC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad7-lower	CAAGCAGAAGACGGCATACGAGAT**GATCTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	

3'Ad8-lower	CAAGCAGAAGACGGCATACGAGAT**TCAAGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad9-lower	CAAGCAGAAGACGGCATACGAGAT**CTGATC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad10-lower	CAAGCAGAAGACGGCATACGAGAT**AAGCTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad11-lower	CAAGCAGAAGACGGCATACGAGAT**GTAGCC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad12-lower	CAAGCAGAAGACGGCATACGAGAT**TACAAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad13-lower	CAAGCAGAAGACGGCATACGAGAT**TTGACT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad14-lower	CAAGCAGAAGACGGCATACGAGAT**GGAACT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad15-lower	CAAGCAGAAGACGGCATACGAGAT**TGACAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad16-lower	CAAGCAGAAGACGGCATACGAGAT**GGACGG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad17-lower	CAAGCAGAAGACGGCATACGAGAT**CTCTAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad18-lower	CAAGCAGAAGACGGCATACGAGAT**GCGGAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad19-lower	CAAGCAGAAGACGGCATACGAGAT**TTTCAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad20-lower	CAAGCAGAAGACGGCATACGAGAT**GGCCAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad21-lower	CAAGCAGAAGACGGCATACGAGAT**CGAAAC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad22-lower	CAAGCAGAAGACGGCATACGAGAT**CGTACG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	

3'Ad23-lower	CAAGCAGAAGACGGCATACGAGAT**CCACTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad24-lower	CAAGCAGAAGACGGCATACGAGAT**GCTACC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad25-lower	CAAGCAGAAGACGGCATACGAGAT**ATCAGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad26-lower	CAAGCAGAAGACGGCATACGAGAT**GCTCAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad27-lower	CAAGCAGAAGACGGCATACGAGAT**AGGAAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad28-lower	CAAGCAGAAGACGGCATACGAGAT**CTTTTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad29-lower	CAAGCAGAAGACGGCATACGAGAT**TAGTTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad30-lower	CAAGCAGAAGACGGCATACGAGAT**CCGGTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad31-lower	CAAGCAGAAGACGGCATACGAGAT**ATCGTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
3'Ad32-lower	CAAGCAGAAGACGGCATACGAGAT**TGAGTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGA	TruGrade	
qPCR_F	CGACGCTCTTCCGATCT	desalted	
PCR_F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCC	HPLC	
PCR_R	CAAGCAGAAGACGGCATACGA	HPLC	

Note: primer sets for rRNA contamination check qPCR are for human samples only. These sequences are species-specific. If libraries are being generated from samples from a different species, design appropriate primer sets according to standard qPCR guidelines and test their efficiency before use.

Note: How many 3' adaptor oligo pairs you will need depends on how many samples you wish to pool in a single sequencing run. If performing this experiment with less than 32 samples, the number of adaptor oligo pairs you will need to purchase reduces accordingly.

CRITICAL STEP: Although the variable portion of the 5' adaptor (in bold in the table above) is not used in this assay for indexing/barcoding purposes, it is highly recommended that multiple 5' adaptors be used in a sequencing pool to increase diversity of the sequence in the crucial initial 5 cycles the sequencer requires for cluster registration. The combination of adaptors used should be specifically chosen to maximise base diversity at each position. If this

guideline is not followed, the proportion of PhiX spike-in to the final sequencing library should be increased to avoid a poor quality sequencing run.

H. REAGENT SETUP

Tris-HCl, 1M (pH 7.0)

Adjust the pH of Tris with HCl. (HCl is poisonous. When you adjust the pH with HCl stock solution, handle using appropriate safety equipment.)

Tris-HCl, 10 mM (pH 8.5)

Adjust the pH of Tris with HCl.

Sorbitol (3.3 M) / (0.66 M) trehalose mix

Saturate 8.02 g of trehalose and 17.8 g of sorbitol in 30 ml of water and autoclave the mixture at 121 °C for 30 min. Store at room temperature (15–25 °C) for up to 1 year or store at − 20 °C in aliquots for up to 5 years. (Trehalose and sorbitol should be of high quality, and essentially free of heavy metals that could cause nucleic acid degradation.)

NaIO4 for oxidation of the diol groups, 250 mM

Dissolve 0.053 g of NaIO4 in 1 ml of water. (The solution should be freshly prepared at room temperature before use and kept in the dark.)

Biotin (long arm) hydrazide for biotinylation, 15 mM

Dissolve 0.0038 g of biotin (long arm) hydrazide in 675 µl of water at room temperature. (The solution should be freshly prepared before use and kept in the dark. Biotin will not dissolve immediately in the water, and thus continuous mixing is necessary until the biotin is dissolved.)

E. coli tRNA, 20 µg/µl

Dissolve 30 mg of E. coli tRNA lyophilized powder in 400 µl of water and add 45 µl of 10× RQ1 DNase buffer and 30 µl of RQ1 RNase-free DNase. Incubate at 37 °C for 2 h. Add 10 µl of 0.5 M EDTA (pH 8.0), 10 µl of 10% (wt/vol) SDS and 10 µl of 10 ng/ml proteinase K to the tRNA solution. Incubate at 45 °C for 30 min. Extract with 500 µl of phenol/chloroform and centrifuge at 15,000 r.p.m. for 3 min at room temperature. Collect the aqueous phase and extract with 500 µl of chloroform. Centrifuge again at 15,000 r.p.m. for 3 min at room temperature. Collect the aqueous phase and add 25 µl of 5 M NaCl and 525 µl of isopropanol. Centrifuge at 15,000 r.p.m. for 5 min at room temperature. Remove the supernatant and add 900 µl of 80% (vol/vol) ethanol to the tRNA pellet. Centrifuge at 15,000 r.p.m. for 5 min at room temperature. Repeat the ethanol wash and centrifugation, and then discard the supernatant and dissolve the tRNA pellet in 1.5 ml of water. Divide into 20µl aliquots and store at − 20 °C for up to 5 years.

Wash buffer 1

Mix 45 ml of 5 M NaCl and 5 ml of 0.5 M EDTA (pH 8.0). Store at room temperature for up to 1 year.

Wash buffer 2

Mix 3 ml of 5 M NaCl, 100 µl of 0.5 M EDTA (pH 8.0) and 46.9 ml of water. Store at room temperature for up to 1 year.

Wash buffer 3

Mix 1 ml of 1 M Tris-HCl (pH 8.5), 100 µl of 0.5 M EDTA (pH 8.0), 25 ml of 1 M sodium acetate (pH 6.1), 2 ml of 10% (wt/vol) SDS and 21.9 ml of water. Store at room temperature for up to 1 year. (If the room temperature drops, 10% (wt/vol) SDS in wash buffer 3 may form crystals. In this case, dissolve crystallized SDS in a water bath at 37 °C before use.)

Wash buffer 4

Mix 500 µl of 1 M Tris-HCl (pH 8.5), 100 µl of 0.5 M EDTA (pH 8.0), 25 ml of 1 M sodium acetate (pH 6.1) and 24.4 ml of water. Store at room temperature for up to 1 year.

5' adaptors

1. Prepare a 50uM solution of each 5' adaptor in 1 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA (pH 8.0).

2. Mix 6 µl of each specific 5' upper adaptor, 6 µl of each specific 5' lower adaptor, 3 µl of 1 M NaCl and 15 µl of nuclease-free water. Final concentration is 10uM each in final volume of 30uL.

CRITICAL STEP: Upper and lower adaptors with matching specific barcode sequences are combined to form a double stranded product with partial single-strand random protruding ends, which ligate to the terminal end of the cDNA.

3. To carry out the annealing reaction, incubate the adaptor reaction solutions using the following conditions: 95 °C, 5 min; − 0.1 °C /s down to 83 °C; 5 min at 83 °C; − 0.1 °C/s down to 71 °C; 5 min at 71 °C; − 0.1 °C s/ down to 59 °C; 5 min at 59 °C; − 0.1 °C/s to 47 °C; 5 min at 47 °C; − 0.1 °C/s to 35 °C; 5 min at 35 °C; − 0.1 °C/s to 23 °C; 5 min at 23 °C; − 0.1 °C/s to 11 °C, and then hold at 11 °C (annealing is considered complete when the temperature of the sample reaches 11 °C).

CRITICAL STEP: Annealing takes place by slowly cooling the adaptors at the described temperature.

4. The final annealed adaptor solutions can be kept at 4 °C for 1 month, but for long-term storage they should be frozen at − 20 °C and can be kept for up to 5 years.

3' adaptors

1. Prepare a 50uM solution of each 3' adaptor in 1 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA (pH 8.0).

2. Mix 6 µl of each specific 3' upper adaptor, 6 µl of each specific 3' lower adaptor, 3 µl of 1 M NaCl and 15 µl of nuclease-free water. Final concentration is 10uM each in final volume of 30uL.

CRITICAL STEP: Upper and lower adaptors with matching specific barcode sequences are combined to form a double stranded product with partial single-strand random protruding ends, which ligate to the terminal end of the cDNA.

3. To carry out the annealing reaction, incubate the adaptor reaction solutions using the following conditions: 95 °C, 5 min; − 0.1 °C/s down to 83 °C; 5 min at 83 °C; − 0.1 °C/s down to 71 °C; 5 min at 71 °C; − 0.1 °C/s down to 59 °C; 5 min at 59 °C; − 0.1 °C/s to 47 °C; 5 min at 47 °C; − 0.1 °C/s to 35 °C; 5 min at 35 °C; − 0.1 °C/s to 23 °C; 5 min at 23 °C; − 0.1 °C/s to 11 °C, and then hold at 11 °C (annealing is considered complete when the temperature of the sample reaches 11 °C).

CRITICAL STEP: Annealing takes place by slowly cooling the adaptors at the described temperature.

4. Dilute adaptors to a concentration of 2.5uM by adding 90uL of 100mM NaCl.

5. The final annealed adaptor solutions can be kept at 4 °C for 1 month, but for long-term storage they should be frozen at − 20 °C and can be kept for up to 5 years.

I. PROCEDURE

CRITICAL STEP: The procedure described here is for a single sample. However, the protocol is commonly performed using multiple samples, including preparation of CAGE libraries with multipipettes. In this case, where appropriate, prepare a master mix of reagents to avoid technical bias.

Workflow Outline:

Day1
- Step 1 - 1st strand cDNA synthesis
- Step 2 - Oxidation
- Step 3 – Biotinylation

Day2
- Step 4 - RNase I digestion
- Step 5 - Preparation of Streptavidin beads
- Step 6 - Cap-trapping and Releasing cDNA
- Step 7 - cDNA Q.C
- Step 8 - Single Strand Linker Ligation

Day3
- Step 9 – AMPure purification
- Step 10 - 3' Linker Ligation

Day4
- Step 11 – AMPure purification
- Step 12 – SAP/USER treatment
- Step 13 – qPCR analysis for pooling

Day5
- Step 14 - PCR
- Step 15 – Exonuclease I treatment
- Step 16 - final Q.C

Day 1

◆Step 1<a> - 1st strand cDNA synthesis (1 hour 30 min)

Purpose: Synthesize the 1st strand cDNA by reverse transcription reaction with random primers using total RNA as a template.

1. Thaw RNA on ice.
2. Dispense 5 μl of total RNA at 1 μg/ μl (5μg total) into 8-strip tubes.
   Note: Perform ethanol precipitation or Eppendorf Speed-vac concentration in case RNA concentration is too low. However, DO NOT use glycogen for ethanol precipitation (as this adversely effects biotinylation reaction). When the concentrator is used, DO NOT dry the sample completely.
3. Dispense 2.5 μl/sample of 1mM RT primer ($TCT(N)_6$) into the sample tube that contains RNA. Spin down the tubes in a centrifuge to collect the solution to the bottom. Place the plate on ice until use.
4. Set the sample tubes in a thermal cycler that is set at 65°C. Incubate for 5 min with lid temperature at 75°C.
5. After incubation in step 4, immediately place the tubes on ice and stand for 1min.
6. Prepare RT enzyme premix [Table 1] in a 1.5 ml tube. Mix well by pipetting slowly 10 times. Spin down to collect solution to the bottom of the tube in the tabletop centrifuge. Place the tube on ice until just before use.

Note: Pipette slowly when aliquoting Trehalose / Sorbitol because it is high in viscosity. After dispensing, mix well by pipetting.

Table 1 : RT enzyme premix

Reagent	1 sample
H_2O	9.38 µl
5X SS III reaction buffer	7.5 µl
10mM dNTP	1.87 µl
Sorbitol/Trehalose	7.5 µl
Superscript III (200U/ul)	3.75 µl
Total volume	30 µl

7. Dispense 30 µl/sample of RT enzyme premix into the sample tubes.
8. Set the pipette to 30 µl and mix the reaction mixture well by pipetting 10 times.
9. Spin down the tubes to collect the solution to the bottom of the tubes.
10. Incubate RNAClean XP for 30 min at room temperature and pre warm H_2O at 37ºC.
11. Set the sample tube in the thermal cycler and carry out reverse transcription according to Table 2.

Table 2: CAGE_RT program (lid temp: 70ºC)

Temperature	Time
25ºC	30 sec
42ºC	30 min
50ºC	10 min
56ºC	10 min
60ºC	10 min
4ºC	∞

12. After the reaction is completed, spin down the samples to collect the solution to the bottom of the tubes.
13. Place the sample on ice or -80°C until proceeding to step 1 <b>.

◆Step 1<b> cDNA/RNA purification with RNAClean XP (1 hour 30 min)

Purpose: Remove unreacted primers and exchange buffer.

1. Add 67.5 µl/sample of well-mixed RNAClean XP beads into the sample tubes. Set the pipette to 85 µl and mix well by pipetting 10 times.
2. Incubate for 30 min at room temperature, mixing every 10 min by pipetting.
3. After incubation, set the sample tubes at the magnetic bar and stand for 5min or until solution is clear.
   Note: leave the lid open while the plate is set at the magnetic bar.
4. Prepare 70% (vol/vol) ethanol for the washing step.
   Note: Make up 70% ethanol fresh before use.
5. Remove the supernatant by pipetting.

Note: When removing the supernatant leave a small amount of the supernatant (approximately 2 µl), which can help avoid aspirating beads. In order to avoid losing sample, be sure not to aspirate beads in the pipette tip together with the solution.

6. Add 200 µl of 70% ethanol into each well and leave for 30 sec.
   Note: Carry out the ethanol wash with the tubes on the magnetic bar.
   Note: Pipetting is not required after adding ethanol.
   Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
7. Remove the supernatant in the same way as step 5.
   Note: Remove ethanol as much as possible.
8. Repeat step 6 and 7 (wash twice total)
9. Spin down the tubes, pipette out the remaining ethanol and air-dry the beads for up to 5 min at room temperature. Do not overdry.
10. Remove the sample tubes from the magnetic bar. Add 40 µl/sample of 37°C-H_2O and suspend the beads by pipetting 60 times.
11. Set the sample tubes in a thermal cycler and incubate for 10 min at 37°C.
12. Spin down the tubes to collect the scattered beads to the bottom, if necessary.
13. Set the sample tubes at the magnetic bar and stand for 5 min or until solution is clear.
14. Collect the supernatant (up to 40µl) with a pipette and transfer it to new 8-strip sample tubes.
    To remove the ethanol remaining in the eluted sample from the washing process, set the sample tubes in the thermal cycler without the cap of the tube and incubate for 5 min at 37°C. In case ethanol droplets were still visible after step 9 (possible if multiwell plate is used) incubate for 10 min at 37°C. Note: Place the lid of a tip box that has been wiped with RNAseZap over the plate to avoid contamination with dust.
15. Close the cap of the sample tubes.
16. Spin down the tubes in the tabletop centrifuge to collect the solution to the bottom.
17. Keep the cDNA on ice until the next step.

◆Step 2<a> Oxidation (1hr)

Purpose: mRNA contains two diol groups in the cap structure at the 5'-end and ribose at the 3'-end.

Oxidize with sodium periodate ($NaIO_4$).

CRITICAL STEP: Sodium periodate ($NaIO_4$) is sensitive to light. Protect from light by wrapping with aluminium foil and by closing the lid of the ice bucket during the reaction.

1. Prepare oxidation reaction mixture on ice according to Table 3.

Table 3 : Oxidation reaction mixture

Reagent	1 sample
Sample	40 µl
1M NaOAc (pH 4.5)	2 µl
250 mM $NaIO_4$	2 µl
Total volume	44 µl

2. Set the scale of the pipette to 40 µl and mix well by pipetting 10 times.
3. Spin down to collect the solution to the bottom of the tube.
4. Incubate for 45 min on ice protected from light.

CRITICAL STEP: This reaction must be done under light-blocking conditions.

CRITICAL STEP: Strictly keep to the reaction time to avoid over-reaction.

5. Incubate RNAClean XP for 30 min at room temperature and pre warm H_2O at 37°C
6. After 45min incubation, add 2 µl/sample of 40% glycerol into the sample tubes. Set the pipette to 40 µl and mix well by pipetting 10 times.
   Note: Slowly pipette 40% glycerol because it is a high-viscosity reagent.
7. Add 14 µl/sample of 1 M Tris-HCl (pH 8.5) into the sample tubes to bring the pH above 5.6 (total volume 60 µl). Set the pipette to 40 µl and mix well by pipetting 10 times.

◆Step 2 <b> cDNA/RNA purification with RNA clean XP (1 hour 30 min)

1. Add 108 µl/sample of well-mixed RNAClean XP beads into the sample tubes. Set the pipette to 150 µl and mix well by pipetting 10 times.
2. Incubate for 30 min at room temperature, mixing every 10 min by pipetting.

Note: In parallel with this procedure, prepare the 15 mM biotin hydrazide (long arm) solution. Wrap in aluminium foil to protect from light.

3. After incubation, set the sample tubes at the magnetic bar and stand for 5min or until solution is clear.
4. Prepare 70% (vol/vol) ethanol for the washing step.
   Note: Make up 70% ethanol fresh before use.
5. Remove the supernatant by pipetting.
   Note: When removing the supernatant leave a small amount of the supernatant (approximately 2 µl), which can help avoid aspirating beads. In order to avoid losing sample, be sure not to aspirate beads in the pipette tip together with the solution.
6. Add 200 µl of 70% ethanol into each well and leave for 30 sec.
   Note: Carry out the ethanol wash with the tubes on the magnetic bar.
   Note: Pipetting is not required after adding ethanol.
   Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
7. Remove the supernatant in the same way as step 5.
   Note: Remove ethanol as much as possible.
8. Repeat step 6 and 7 (wash twice total)
9. Spin down the tubes, pipette out the remaining ethanol and air-dry the beads for up to 5 min at room temperature. Do not overdry.
10. Remove the sample tubes from the magnetic bar. Add 40 µl/sample of 37°C-H_2O and suspend the beads by pipetting 60 times.
11. Set the sample tubes in a thermal cycler and incubate for 10 min at 37°C.
12. Spin down the tubes to collect the scattered beads to the bottom, if necessary.
13. Set the sample tubes at the magnetic bar and stand for 5 min or until solution is clear.
14. Collect the supernatant (up to 40µl) with a pipette and transfer it to new 8-strip sample tubes.
    To remove the ethanol remaining in the eluted sample from the washing process, set the sample tubes in the thermal cycler without the cap of the tube and incubate for 5 min at 37°C. In case ethanol droplets were still visible after step 9 (possible if multiwell plate is used) incubate for 10 min at 37°C. Note: Place the lid of a tip box that has been wiped with RNAseZap over the plate to avoid contamination with dust.
15. Close the cap of the sample tubes.

16. Spin down the tubes in the tabletop centrifuge to collect the solution to the bottom.
17. Keep the cDNA on ice until the next step.

◆Step 3 Biotinylation (16 h, overnight)

Purpose: Bind biotin (long arm) hydrazide to oxidised diol groups.

CRITICAL STEP: Biotin (long arm) hydrazide is sensitive to light. Protect from light by wrapping with aluminium foil and by closing the lid of the thermal cycler during the reaction.

1. Prepare biotinylation reaction mixture on ice according to Table 4.

Table 4 : Biotinylation reaction mixture

Reagent	1 sample
Purified cDNA/RNA from Step 2<b>.17	40 µl
1M NaOAc (pH 6.0)	4 µl
15mM Biotin Hydrazide (long arm)	13.5 µl
Total volume	57.5 µl

2. Set the pipette to 40 µl and mix well by pipetting 10 times.
3. Spin down the sample tubes to collect the solution to the bottom.
4. Set the tubes in a thermal cycler. Incubate for 16 hrs (overnight) at 23℃ under light-blocking conditions (lid temp: 25ºC).

Day 2

◆Step 4<a> RNase I treatment (1hr)

Purpose: Digest single strand RNA using RNase I. This step selects for cDNA that has extended to the 5'-end of capped RNA.

1. Prepare RNase reaction mixture on ice according to Table 5.

Table 5 : RNase reaction mixture

Reagent	1 sample
Biotinylation reaction from Step 3.4	57.5 µl
Tris-HCl (1M, pH 8.5)	6 µl
EDTA (0.5M, pH 8.0)	1 µl
RNase One ribonuclease (10U/ul)	5 µl
Total volume	69.5 µl

2. Set the pipette to 60 µl and mix well by pipetting 10 times.
3. Spin down the tubes to collect the solution to the bottom.
4. Set the sample tubes in a thermal cycler and incubate according to Table 6.

Table 6: RNAse I incubation (lid temp: 75ºC)

Temperature	Time
37 °C	30 min
65 °C	5 min

5. Cool on ice immediately for at least 2 mins.
6. Keep on ice until proceeding to Step 4<b>.

◆Step 4<b> cDNA/RNA purification with RNAClean XP (1 hour 30 min)

1. Add 125 µl/sample of well-mixed RNAClean XP beads into the sample tubes. Set the pipette to 150 µl and mix well by pipetting 10 times.
2. Incubate for 30 min at room temperature, mixing every 10 min by pipetting.

Note: In parallel with this procedure, begin preparation of the streptavidin beads (Step 5<a>, below).

3. After incubation, set the sample tubes at the magnetic bar and stand for 5min or until solution is clear.
4. Prepare 70% (vol/vol) ethanol for the washing step.
   Note: Make up 70% ethanol fresh before use.
5. Remove the supernatant by pipetting.
   Note: When removing the supernatant leave a small amount of the supernatant (approximately 2 µl), which can help avoid aspirating beads. In order to avoid losing sample, be sure not to aspirate beads in the pipette tip together with the solution.
6. Add 200 µl of 70% ethanol into each well and leave for 30 sec.
   Note: Carry out the ethanol wash with the tubes on the magnetic bar.
   Note: Pipetting is not required after adding ethanol.
   Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
7. Remove the supernatant in the same way as step 5.
   Note: Remove ethanol as much as possible.
8. Repeat step 6 and 7 (wash twice total)
9. Spin down the tubes, pipette out the remaining ethanol and air-dry the beads for up to 5 min at room temperature. Do not overdry.
10. Remove the sample tubes from the magnetic bar. Add 40 µl/sample of 37°C-H_2O and suspend the beads by pipetting 60 times.
11. Set the sample tubes in a thermal cycler and incubate for 10 min at 37°C.
12. Spin down the tubes to collect the scattered beads to the bottom, if necessary.
13. Set the sample tubes at the magnetic bar and stand for 5 min or until solution is clear.
14. Collect the supernatant (up to 40µl) with a pipette and transfer it to new 8-strip sample tubes.
    To remove the ethanol remaining in the eluted sample from the washing process, set the sample tubes in the thermal cycler without the cap of the tube and incubate for 5 min at 37°C. In case ethanol droplets were still visible after step 9 (possible if multiwell plate is used) incubate for 10 min at 37°C. Note: Place the lid of a tip box that has been wiped with RNAseZap over the plate to avoid contamination with dust.
15. Close the cap of the sample tubes.
16. Spin down the tubes in the tabletop centrifuge to collect the solution to the bottom.
17. Keep the cDNA on ice until the next step.

◆Step 5<a> Preparation of streptavidin beads (1 hour 30 min)

Purpose: To avoid unspecific biotin-streptavidin binding, coat streptavidin beads with tRNA.

CRITICAL STEP Coating the beads with tRNA before cDNA capture is essential for diminishing nonspecific cDNA/bead interactions and thus reducing contamination with cDNA trancripts that did not reach the cap site, or cDNA from uncapped RNA. Note that E. coli tRNAs are added after the RT reaction and thus these sequences cannot contaminate the CAGE library.

1. Streptavidin beads are precipitated. Mix well to diffuse them by inverting the tube.
2. Dispense 100 μl/sample of streptavidin beads into a 2.0 ml tube.
3. Add 1.5μl/sample of 20 μg/μl tRNA and mix by tapping the tube.
   Note: In the following procedure, this mixture will be described as 'tRNA-streptavidin beads'.
4. Incubate for 30 min on ice (tap the tube every 5 min to diffuse the tRNA-streptavidin beads).
5. After the incubation, centrifuge for 5 sec in a tabletop centrifuge to collect the solution and the tRNA-streptavidin beads to the bottom of the tube.
6. Set the tube at the magnetic bar and stand for 3 min to allow tRNA-streptavidin beads to collect at the wall of the tube.
7. Remove the supernatant by pipetting.
8. Remove the tube from the magnetic bar. Add 50 μl/sample of Wash Buffer 1 and diffuse the tRNA-streptavidin beads by pipetting.
9. Centrifuge the tube in a tabletop centrifuge to collect the solution and the tRNA-streptavidin beads to the bottom.
10. Set the tube at the magnetic bar and stand for 3min to allow tRNA streptavidin beads to collect at the wall of the tube.
11. Remove the supernatant by pipetting.
12. Repeat the steps 8-11 (wash with Wash Buffer 1 twice).
13. Add 80 μl/sample of Wash Buffer 1 and diffuse the tRNA-streptavidin beads by pipetting 10 times.
14. Dispense 80 μl/sample of the mixture into 8-strip tubes.
    Note: The tRNA-streptavidin beads tend to precipitate quickly. Dispense them into tubes immediately after diffusing them by pipetting.
15. Place on ice until just before use.

CRITICAL STEP: Use the samples within 1 hr of preparation, as streptavidin on the tRNA-streptavidin beads is unstable in high salt conditions.

◆Step 6<a> Cap-trapping and releasing cDNA (1 hour 30 min)

Purpose: Bind biotinylated capped RNA to the streptavidin beads and remove unbound transcripts by washing. Collect cDNA that derives from capped RNA through denaturation of cDNA/RNA hybrid with NaOH.

1. Incubate AMPure XP for 30 min at room temperature and prewarm H_2O at 37°C.
2. Add 40 μl of the purified cDNA/RNA from Step 4<b>.17 to 80 μl of washed beads from Step 5<a>.15. Set the scale of the pipette at 110 μl and mix well by pipetting 10 times.
3. Incubate at room temperature for 30 min (pipette thoroughly 10 times every 5 min).
4. After incubation, set the sample tubes at the magnetic bar and stand for 5min.
5. Remove the supernatant by pipetting.

6. Remove the sample tubes from the magnetic bar. Add 150 µl/sample of Wash Buffer 1 and diffuse the tRNA-streptavidin beads by pipetting.
   Note: The Wash Buffer contains Tween20. Avoid bubbles during pipetting
7. Set the sample tubes at the magnetic bar and stand for 3min.
8. Remove the supernatant by pipetting.
9. Remove the sample tubes from the magnetic bar. Add 150 µl/sample of Wash Buffer 2 and diffuse the tRNA-streptavidin beads by pipetting.
10. Set the sample tubes at the magnetic bar and stand for 3min.
11. Remove the supernatant by pipetting.
12. Remove the sample tubes from the magnetic bar. Add 150 µl/sample of Wash Buffer 3 and diffuse the tRNA-streptavidin beads by pipetting.
13. Set the sample tubes at the magnetic bar and stand for 3min.
14. Remove the supernatant by pipetting. Repeat steps 12-14. (Wash with Wash Buffer 3 **twice**).
15. Remove the sample tubes from the magnetic bar. Add 150 µl/sample of Wash Buffer 4 and diffuse the tRNA-streptavidin beads by pipetting.
16. Set the sample tubes at the magnetic bar and stand for 3min.
17. Remove the supernatant by pipetting. Repeat steps 15-17. (Wash with Wash Buffer 4 **twice**).

CRITICAL STEP: It is important to wash multiple times. This helps prevent contamination of noncapped molecules in the final CAGE library.

18. Add 60 µl of 50 mM NaOH solution to the beads and diffuse the tRNA-streptavidin beads by pipetting.
19. Incubate at room temperature for 10 min, with mixing by pipetting every 2-3 min.
20. Set the sample tubes at the magnetic bar and stand for 3min.
21. Transfer 60 µl of supernatant to a new tube and place on ice.
22. Add 12 µl of ice-cold 1 M Tris-HCl (pH 7.0) to the 60 µl of eluant. The total volume is now 72 µl. Keep the cDNA on ice until proceeding to the next step.

◆Step 6<b> cDNA purification with AMpure XP (1 hour 30 min)

1. Add 130 µl/sample of well-mixed AMPure XP beads into the sample tubes. Set the pipette to 180 µl and mix well by pipetting 10 times.
2. Incubate for 30 min at room temperature, mixing every 10 min by pipetting.
3. After incubation, set the sample tubes at the magnetic bar and stand for 5min or until solution is clear.
4. Prepare 70% (vol/vol) ethanol for the washing step.
   Note: Make up 70% ethanol fresh before use.
5. Remove the supernatant by pipetting.
   Note: When removing the supernatant leave a small amount of the supernatant (approximately 2 µl), which can help avoid aspirating beads. In order to avoid losing sample, be sure not to aspirate beads in the pipette tip together with the solution.
6. Add 200 µl of 70% ethanol into each well and leave for 30 sec.
   Note: Carry out the ethanol wash with the tubes on the magnetic bar.
   Note: Pipetting is not required after adding ethanol.
   Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
7. Remove the supernatant in the same way as step 5.
   Note: Remove ethanol as much as possible.

8. Repeat step 6 and 7 (wash twice total)
9. Spin down the tubes, pipette out the remaining ethanol and air-dry the beads for up to 5 min at room temperature. Do not overdry.
10. Remove the sample tubes from the magnetic bar. Add 40 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times.
11. Set the sample tubes in a thermal cycler and incubate for 10 min at 37°C.
12. Spin down the tubes to collect the scattered beads to the bottom, if necessary.
13. Set the sample tubes at the magnetic bar and stand for 5 min or until solution is clear.
14. Collect the supernatant (up to 40µl) with a pipette and transfer it to new 1.5mL tubes.
15. Keep the cDNA on ice until the next step.
16. Transfer 5 µl of sample to a new tube to use for quality control check.

◆Step 7 cDNA QC (1 hour 30 min)

Note: 5 µl aliquot can be stored at 4 °C overnight and QC performed the following day. In this case, move directly to concentration of sample and linker ligation (Step 8).

<QC1> Concentration check by Oligreen assay – Please refer to 'SOP002-02 CAGE Quant-iT Oligreen ssDNA assay', which can be found on the CAGE development page of the GIH website.

The total amount of enriched single strand cDNA obtained may be different depending on the source of the RNA sample. As reference, a few to a few dozen ng of single strand cDNA is appropriate.

Important: average expected yield of cDNA is 15 - 30 ng

<QC2> Enrichment check by qPCR – Please refer 'SOP003-02 CAGE rRNA contamination qPCR', which can be found on the CAGE development page of the GIH website.

Ideally, the Ct difference between ACTB and rRNA genes should be ≤ 4.

◆Step 8 5' adaptor ligation to single-stranded cDNA (16 hour, overnight)

Purpose: Add the 5' adaptor, which is required for sequencing, to the 3' end of cDNA.

1. Concentrate the cDNA using a vacuum concentrator at room temperature in a 1.5ml tube, and then adjust volume to 4 µl with water. Do not over dry and stop when the volume is ≤ 4 µl but there is still liquid remaining.
2. Transfer samples into 8-strip tubes.
3. Add 1.4 µl of a different 5' adaptor (10uM; prepared as above in Reagent Setup) to an empty tube for each cDNA sample.
4. Incubate both adaptor and sample tubes at 65 °C for 5 min. Immediately transfer to ice for 2 min.

CRITICAL STEP: It is important to denature the adaptor and cDNA secondary structure for efficient ligation.

5. Spin down the sample tubes and the adaptor tubes to collect the solution to the bottom of the tube.
6. Add 10 µl of DNA ligation Mighty Mix to each sample tube.
7. Add 1 µl of 5' adaptors to the cDNA mix (total volume 15 µl).
8. Set the pipette to 10 µl and mix well by pipetting at least 20 times.
   Note: Ligation mix is very viscous and difficult to mix. Ensure that it is mixed well.
9. Spin down the tubes to collect the solution at the bottom of the tube.
10. Set the sample tubes in a thermal cycler that is set at 16°C (lid temp: 25°C) and incubate for 16 hrs.

Note : Immediately proceed to the next step after the incubation. When this is not possible, store the samples at -20°C.

CRITICAL STEP: Use differently barcoded 5' adaptors (up to 8 samples; can repeat adaptor use for >8 samples) to improve sequencing metrics.

Day 3

◆Step 9 cDNA purification with 2x AMPure XP clean-up (3 hour)

Purpose: Remove remaining unligated 5' adaptors.

1. Incubate AMPure XP for 30 min at room temperature and prewarm H_2O at 37°C.
2. Add 9 ul of H_2O to the 15 ul of 5' adaptor-ligated samples.
3. Add 43.2 µl/sample of well-mixed AMPure XP into the sample tubes. Set the pipette to 50 µl and mix well by pipetting 10 times.
4. Incubate for 30 min at room temperature, mixing every 10 min by pipetting.
5. After incubation, set the sample tubes at the magnetic bar and stand for 5min or until solution is clear.
6. Prepare 70% (vol/vol) ethanol for the washing step.
   Note: Make up 70% ethanol fresh before use.
7. Remove the supernatant by pipetting.
   Note: When removing the supernatant leave a small amount of the supernatant (approximately 2 µl), which can help avoid aspirating beads. In order to avoid losing sample, be sure not to aspirate beads in the pipette tip together with the solution.
8. Add 200 µl of 70% ethanol into each tube and leave for 30 sec.
   Note: Carry out the ethanol wash with the tubes on the magnetic bar.
   Note: Pipetting is not required after adding ethanol.
   Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
9. Remove the supernatant in the same way as step 7.
   Note: Remove ethanol as much as possible.
10. Repeat step 8 and 9 (wash twice total).
11. Spin down the tubes, pipette out the remaining ethanol and air-dry the beads for up to 5 min at room temperature. Do not overdry.
12. Remove the sample tubes from the magnetic bar. Add 40 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times.
13. Set the sample tubes in a thermal cycler and incubate for 10 min at 37°C.
14. Spin down the tubes to collect the scattered beads to the bottom, if necessary.
15. Set the sample tubes at the magnetic bar and stand for 5 min or until solution is clear.
16. Collect the supernatant (up to 40µl) with a pipette and transfer it to new 8-strip tubes.
17. Keep the cDNA on ice until the next step.
18. Add 72 µl/sample of well-mixed AMPure XP into the sample tubes. Set the scale of pipette at 100 µl and mix well by pipetting 10 times.
19. Incubate for 30 min at room temperature, mixing every 10 min by pipetting.
20. After incubation, set the sample tubes at the magnetic bar and stand for 5min or until solution is clear.
21. Prepare 70% (vol/vol) ethanol for the washing step.
22. Remove the supernatant by pipetting.
    Note: When removing the supernatant leave a small amount of the supernatant (approximately 2 µl), which can help avoid aspirating beads. In order to avoid losing sample, be sure not to aspirate beads in the pipette tip together with the solution.

23. Add 200 µl of 70% ethanol into each tube and leave for 30 sec.
    Note: Carry out the ethanol wash with the tubes on the magnetic bar.
    Note: Pipetting is not required after adding ethanol.
    Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
24. Remove the supernatant in the same way as the step 22.
    Note: Remove ethanol as much as possible.
25. Repeat step 23 and 24 (wash twice total).
26. Spin down the tubes, pipette out the remaining ethanol and air-dry the beads for up to 5 min at room temperature. Do not overdry.
27. Remove the sample tubes from the magnetic bar. Add 40 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times.
28. Set the sample tubes in the thermal cycler and incubate for 10 min at 37°C.
29. Spin down the tubes to collect the scattered beads to the bottom, if necessary.
30. Set the sample tubes at the magnetic bar and stand for 5 min or until solution is clear.
31. Collect the supernatant (up to 40µl) with a pipette and transfer it to new 1.5mL tubes.
32. Keep the cDNA on ice until the next step.

◆Step 10 3' adaptor ligation

Purpose: Add 3' adaptor that is required for sequencing to cDNA.

1. Concentrate the cDNA using a vacuum concentrator at room temperature in a 1.5ml tube, and then adjust volume to 4 µl with water. Do not over dry and stop when the volume is ≤ 4 µl but there is still liquid remaining.
2. Transfer samples into 8-strip tubes.
3. Add 4.4 µl of a different 3' adaptor (2.5uM; prepared as above in Reagent Setup) to an empty tube for each cDNA sample.

CRITICAL STEP: Use a unique barcoded 3' adaptor for each sample to allow pooling of samples for sequencing

4. Incubate sample tubes at 95 °C for 2 min. Immediately transfer to ice for 2 min.
5. At the same time, incubate the 3'adaptor tubes at 65°C for 5 min. Immediately transfer to ice for 2 min.

CRITICAL STEP: It is important to denature the adaptor and cDNA secondary structure for efficient ligation.

6. Spin down the sample tubes and the adaptor tubes to collect the solution at the bottom of the tube.
7. Add 10 µl of DNA ligation Mighty Mix to each sample tube.
8. Add 4 µl of 3' adaptors to the cDNA mix (total volume 18 µl).
9. Set the pipette to 15 µl and mix well by pipetting at least 20 times.
   Note: Ligation mix is very viscous and difficult to mix. Ensure that it is mixed well.
10. Spin down the tubes to collect the solution at the bottom of the tube.
11. Set the sample tubes in a thermal cycler that is set at 16°C (lid temp: 25°C) and incubate for 16 hrs.
    Note : Immediately proceed to the next step after the incubation. When this is not possible, store the samples at -20°C.

Day 4

◆Step 11 AMPure purification (1 hr 30 min)

Purpose: Remove remaining unligated 3' adaptors.

1. Incubate AMPure XP for 30 min at room temperature and prewarm H_2O at 37°C.
2. Add 32.4 µl/sample of well-mixed AMPure XP into the sample tubes. Set the pipette to 40 µl and mix well by pipetting 10 times.
3. Incubate for 30 min at room temperature, mixing every 10 min by pipetting.
4. After incubation, set the sample tubes at the magnetic bar and stand for 5min or until solution is clear.
5. Prepare 70% (vol/vol) ethanol for the washing step.
   Note: Make up 70% ethanol fresh before use.
6. Remove the supernatant by pipetting.
   Note: When removing the supernatant leave a small amount of the supernatant (approximately 2 µl), which can help avoid aspirating beads. In order to avoid losing sample, be sure not to aspirate beads in the pipette tip together with the solution.
7. Add 200 µl of 70% ethanol into each tube and leave for 30 sec.
   Note: Carry out the ethanol wash with the tubes on the magnetic bar.
   Note: Pipetting is not required after adding ethanol.
   Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.
8. Remove the supernatant in the same way as step 6.
   Note: Remove ethanol as much as possible.
9. Repeat step 7 and 8 (wash twice total).
10. Spin down the tubes, pipette out the remaining ethanol and air-dry the beads for up to 5 min at room temperature. Do not overdry.
11. Remove the sample tubes from the magnetic bar. Add 40 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times.
12. Set the sample tubes in a thermal cycler and incubate for 10 min at 37°C.
13. Spin down the tubes to collect the scattered beads to the bottom, if necessary.
14. Set the sample tubes at the magnetic bar and stand for 5 min or until solution is clear.
15. Collect the supernatant (up to 40µl) with a pipette and transfer it to new 8-strip tubes.
16. Keep the cDNA on ice until the next step.

◆Step 12<a> SAP and USER treatment

Purpose: Remove phosphate group from adaptors and digest dUTP contained in 3'adaptor up strand.

1. Prepare the SAP pre-mixture according to Table 7. Place on ice until just before use.

Table 7 : SAP mixture

Reagent	1 sample
H2O	4 µl
10X SAP buffer	5 µl
SAP (1U/ul)	1 µl
Total volume	10 µl

2. Add 10 µl/sample of SAP pre-mixture prepared in step 1 to the sample tubes. Set the pipette to 40µl and mix well by pipetting 10 times.
3. Spin down the tubes to collect the solution to the bottom of the tubes.
4. Set the sample tubes in a thermal cycler that is programmed with the SAP program in Table 8 and start incubation.

Table 8 : SAP program

Temperature	Time
37°C	30 min
65°C	15 min
4°C	∞

5. Place the sample on ice until USER treatment starts.
6. Add 2 µl/sample of USER enzyme to the sample tubes after SAP treatment and mix well by pipetting.
7. Spin down the tubes to collect the solution to the bottom of the tubes.
8. Set the sample tube in the thermal cycler that is programmed with the USER program in Table 9 and start incubation.
   Note: After the step at 95°C for 5min, transfer the tubes to ice to rapidly cool it.
   Note: After incubation at 95°C, the tubes and caps become very hot. Remove them from the thermal cycler when it comes to around 60°C to avoid touching the caps and getting burned.
9. Incubate AMpure XP for 30 min at room temperature and pre warm H_2O at 37°C.

Table 9 : USER program

Temperature	Time
37°C	30 min
95°C	5 min
On ice	2min

◆Step 12<b> Ampure purification (1hr 30min)

1. Add 93.6 µl/sample of well-mixed AMPure XP into the sample tubes. Set the pipette to 140 µl and mix well by pipetting 10 times.
2. Incubate for 30 min at room temperature, mixing every 10 min by pipetting.
3. After incubation, set the sample tubes at the magnetic bar and stand for 5min or until solution is clear.
4. Prepare 70% (vol/vol) ethanol for the washing step.
   Note: Make up 70% ethanol fresh before use.
5. Remove the supernatant by pipetting.
   Note: When removing the supernatant leave a small amount of the supernatant (approximately 2 µl), which can help avoid aspirating beads. In order to avoid losing sample, be sure not to aspirate beads in the pipette tip together with the solution.
6. Add 200 µl of 70% ethanol into each tube and leave for 30 sec.
   Note: Carry out the ethanol wash with the tubes on the magnetic bar.

Note: Pipetting is not required after adding ethanol.
Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.

7. Remove the supernatant in the same way as step 5.
   Note: Remove ethanol as much as possible.
8. Repeat step 6 and 7 (wash twice total).
9. Spin down the tubes, pipette out the remaining ethanol and air-dry the beads for up to 5 min at room temperature. Do not overdry.
10. Remove the sample tubes from the magnetic bar. Add 40 µl/sample of 37°C-H2O and suspend the beads by pipetting 60 times.
11. Set the sample tubes in a thermal cycler and incubate for 10 min at 37°C.
12. Spin down the tubes to collect the scattered beads to the bottom, if necessary.
13. Set the sample tubes at the magnetic bar and stand for 5 min or until solution is clear.
14. Collect the supernatant (up to 40µl) with a pipette and transfer it to new labelled 1.5mL tubes.
15. Keep the cDNA on ice until the next step.

◆Step 13 qPCR analysis for pooling normalisation (2 hour 30 min)

Purpose: Relative quantification of cDNA molecules with both 5' and 3' adaptors successfully ligated to allow pooling of an equal concentration of sequencing-capable DNA samples.

1. Prepare qPCR master mix.

Table 10: qPCR master mix

Reagent	1 sample
2X ABI SYBR green mix	5 µl
qPCR_F + PCR_R primer mix (9 µM each)	1 µl
Total volume	6 µl

2. Make 1 in 100 and 1 in 500 dilutions of cDNA sample as template.
3. Dispense 4 µl of each sample dilution into an appropriate well of 96 well plate, in triplicate.
4. Add 6 µl of qPCR master mix per well.
5. Seal plate with optically-clear film, mix by vortexing, and spin down to collect sample in the bottom of the tube.
6. Perform qPCR analysis with the following cycling conditions.

Table 11: qPCR program

Temperature	Time	Cycles
95 °C	**10 min**	1
95 °C	15 sec	40
60 °C	1 min 30 sec	
Melt : 65 °C – 95 °C (optional)		

7. Enter the Ct values for each sample into a 'CAGE sample pooling template' file, which can be found on the CAGE development page of the GIH website. Instructions for completing this spreadsheet can be found on Sheet 1 'Instructions for use'. Depending on the number of samples and the experimental setup, determine the optimal pooling combination of 4-6 samples per pool and calculate volumes required for pooling of equal quantities of each library per pool.

CRITICAL STEP: Your preferred strategy for assigning samples to pools will depend on your particular experiment. If you wish to compare results from all samples in your sequencing run, you may wish to ensure all pools are of approximately equal concentrations, and will therefore require the same number of PCR cycles. However, this may result in you using only a small proportion of some of the higher-concentration samples. If your samples are from multiple experiments, and the concentrations of your samples are different between experiments, you may wish to pool by experiment to avoid discarding the majority of some samples and therefore needing to perform unnecessary extra PCR cycles. If you have multiple replicates, you may wish to ensure that each replicate is in a different pool to reduce the possibility of batch effects. Combine your samples in whichever way makes the most sense for your experiment.

8. Pool 4-6 samples into a new 1.5 ml tube according to this calculation.
9. Concentrate the cDNA using a vacuum concentrator at room temperature in a 1.5ml tube, and then adjust volume to 40 µl with nuclease-free water. Do not over dry and stop when the volume is ≤ 40 µl.

Day 5

◆Step 14<a> Initial PCR cycles

1. Prepare PCR master mix for each pool according to Table 12 below.

Table 12 : PCR master mix

Reagent	1 sample
H2O	34 µl
5X High fidelity Phusion buffer	40 µl
10 mM dNTPs	4 µl
10 uM PCR_F primer	40 µl
10 uM PCR_R primer	40 µl
Sample (from step 13)	40 µl
Phusion	2 µl
Total volume	200 µl

2. Divide master mix into 4 tubes/pool (50 µl/tube).
3. Place into a thermocycler and run limited cycle PCR according to Table 13 below.

Table 13: PCR program

Temperature	Time	Cycles
98 °C	30 sec	1
98 °C	10 sec	4
66 °C	30 sec	

72 °C	2 min	
4 °C	Hold	

4. Place PCR reaction tubes on ice and proceed directly to Step 14<b>.

◆Step 14<b> Cycle-check qPCR

Purpose: Determine how many additional PCR cycles are required to give a final library concentration of 10-20nM.

1. Prepare qPCR master mix according to Table 14 below.

Table 14 : qPCR master mix

Reagent	1 pool (4 PCR reactions)
KAPA SYBR green master mix	20 µl
10 uM PCR_F primer	4 µl
10 uM PCR_R primer	4 µl
H2O	8 µl
Total volume	36 µl

2. Dispense 9 µl/well (4 wells/pool) into an appropriate well of a 96 well plate (for <=24 samples) or 384 well plate (for >24 samples).
3. Add 1 µl from each replicate PCR tube to a different well containing qPCR mix.
4. Seal plate with optically-clear film, mix by vortexing, and spin down to collect sample in the bottom of the tube.
5. Set the plate in a qPCR machine and perform cycling according to Table 15.

Table 15: cycle-check qPCR program

Temperature	Time	Cycles
95 °C	5 min	1
95 °C	30 sec	25
60 °C	2 min	

6. From the amplification curve, take Ct that corresponds to 75% of maximum Rn for each sample. Remove outliers and average replicates of the same pool.
7. Subtract 5 cycles to give the number of additional cycles required to give the desired quantity of product for sequencing.

CRITICAL STEP: Depending on your experimental setup, you may choose to keep the total PCR cycles the same for all pools. This may mean amplifying some pools for one or two cycles more or less than indicated by the qPCR result. This is usually not a problem, although if the difference is >2 cycles there is a risk of generating either over-amplified or very low concentration libraries.

◆Step 14<c> Final PCR amplification

1. Return the PCR reaction tubes from Step 14<a> to the thermocycler.
2. Run optimal number of cycles that has been determined from Step 14<b>.

◆Step 15<a> Exonuclease I treatment

1. Pool all four of the PCR reactions belonging to the same sample from Step 14<c>, each one containing 49 µl, into one 1.5 ml Lo-Bind microcentrifuge tube.
2. Add 1.3 µl of Exonuclease I (20U/µl) to each tube containing 196 µl of PCR reaction.
3. Mix well by pipetting.
4. Incubate at 37 °C for 30 min.

◆Step 15<b> Purification with Qiagen MinElute PCR purification kit

Purpose: Purify Exonuclease I treated CAGE library pool using the Qiagen MinElute PCR purification kit, following the manufacturer's instructions.

1. Add 1000 µl of PB buffer to each pool and mix well.
2. Transfer each pool + buffer mix into 2 columns (600 µl each).
3. Centrifuge at 13, 000 rpm for 1 min at room temperature and discard flow-through
4. Wash the column with 750 µl of Buffer PE and discard flow-through.
5. Centrifuge the column for an additional 1 min at maximum speed.
6. Place the MinElute column in a clean 1.5 ml Lo-Bind microcentrifuge tube.
7. To elute DNA, add 10 µl of EB buffer, let the column stand for 1 min, then centrifuge for 1 min.
8. Pool the elution of the 2 columns of same library into 1 tube (total volume is 20 µl).

◆Step 15<c> cDNA purification with AMPure XP

1. Incubate AMpure XP for 30 min at room temperature and pre warm H_2O at 37°C.
2. Measure volume of pooled elution and make up to 40 µl with H_2O.
3. Add 40 µl/sample of well-mixed AMPure XP into the sample tubes. Set the pipette to 70 µl and mix well by pipetting 10 times.
4. Incubate for 30 min at room temperature, mixing every 10 min by pipetting.
5. After incubation, set the sample tubes at the magnetic bar and stand for 5min or until solution is clear.
6. Prepare 70% (vol/vol) ethanol for the washing step.
   Note: Make up 70% ethanol fresh before use.
7. Remove the supernatant by pipetting.
   Note: When removing the supernatant leave a small amount of the supernatant (approximately 2 µl), which can help avoid aspirating beads. In order to avoid losing sample, be sure not to aspirate beads in the pipette tip together with the solution.
8. Add 200 µl of 70% ethanol into each tube and leave for 30 sec.
   Note: Carry out the ethanol wash with the tubes on the magnetic bar.
   Note: Pipetting is not required after adding ethanol.

Note: It makes the operation easier to dispense 70% ethanol into clean reservoir in advance when multi-channel pipette is used.

9. Remove the supernatant in the same way as step 7.
   Note: Remove ethanol as much as possible.
10. Repeat step 8 and 9 (wash twice total).
11. Spin down the tubes, pipette out the remaining ethanol and air-dry the beads for up to 5 min at room temperature. Do not overdry.
12. Remove the sample tubes from the magnetic bar. Add 20 µl/sample of 37°C-H_2O and suspend the beads by pipetting 60 times.
13. Set the sample tubes in a thermal cycler and incubate for 10 min at 37°C.
14. Spin down the tubes to collect the scattered beads to the bottom, if necessary.
15. Set the sample tubes at the magnetic bar and stand for 5 min or until solution is clear.
16. Collect the supernatant (up to 20µl) with a pipette and transfer it to new labelled 1.5mL tubes.
17. Aliquot 3µL for QC, and store remainder of library pool at -20°C.

Note: It is possible a significant amount of adaptor dimer (130bp) might still be present after AMPure purification. These smaller fragments can bind to the Illumina flow cell and will preferentially cluster, wasting a significant proportion of reads from a sequencing run. If adaptor dimer is visible on the BioAnalyzer QC performed in Step 16 (below), repeat AMPure purification as per Step 15<c> to remove.

◆Step 16 Final QC – concentration check

<QC1> Check the size distribution of the final product using the BioAnalyser High-Sensitivity DNA kit at a 1/5 dilution. Use region size 150bp-9000bp. If adaptor dimer (around 130 bp) still appears after purification with AMPure, perform additional AMPure purification as per Step 15<c>.

<QC2> Check the DNA concentration using KAPA library quantification kit according to 'SOP004-02 CAGE KAPA library quant qPCR', which is available on the CAGE development page of the GIH website.

Concentration is expected to be ~10-20nM.

1. Obtain final concentration of library using Ct values and standard curve from qPCR and library size from BioA analysis.
2. Pool libraries using equimolar amounts and proceed to sequencing.

Sequencing recommendations using Illumina NextSeq instrument -

Sequencing depth: > 10 million reads/sample (~36 samples/High Output run to allow for pooling discrepancies)
Read configuration: 1x76bp, 6bp single index
Loading concentration: 2.1pM
PhiX spike-in: 5%

J. WORKED EXAMPLE

RNA samples used for CAGE pilot project (ESC1041,1067,1271,1369) have been aliquoted and are available as a positive control for QC testing. Data generated with these samples can be compared to that generated during the pilot project.

Basic QC data from the pilot project are as follows:

<ssDNAQC1> Check concentration by Oligreen assay:

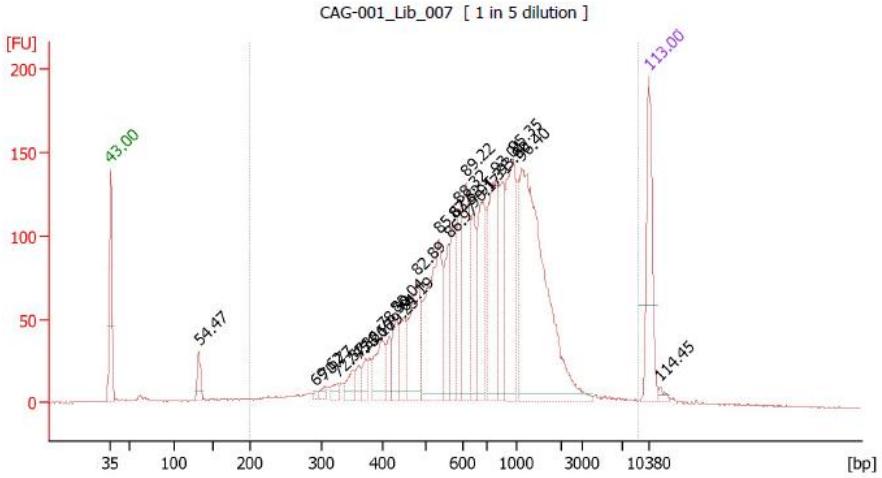
	ng/ml	X100 for dilution factor	pg/ul	Total ssDNA (pg) in 50 ul	Total ng/50µL
ESC1041	4.575	457.5	457.5	22875	22.88
ESC1067	4.233	423.3	423.3	21165	21.17
ESC1271	4.238	423.8	423.8	21190	21.19
ESC1369	3.459	345.9	345.9	17295	17.23

<ssDNAQC2> Check rRNA contamination by qPCR:

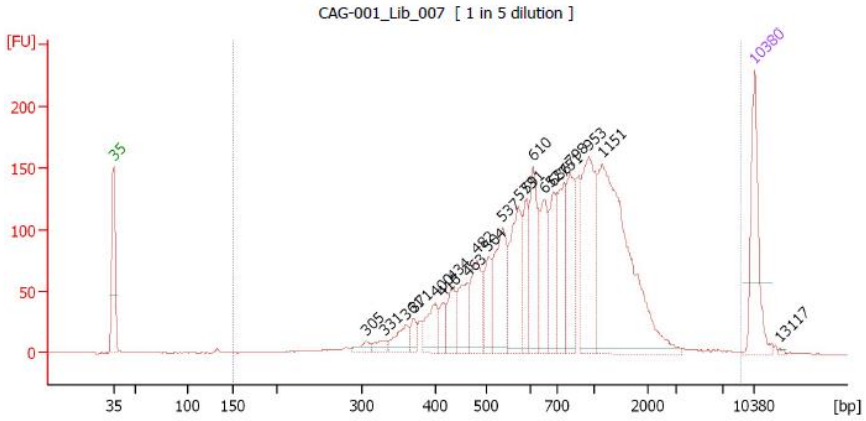
	Mean Ct of rRNA	Mean Ct of ACTB	Delta Ct (ACTB-rRNA)
ESC1041	12.885	15.467	2.582
ESC1067	12.432	14.925	2.493
ESC1271	12.367	14.933	2.566
ESC1369	12.565	15.15	2.585

<LibraryQC1> Check the size distribution of the final product

1) QC after PCR amplification (5 cycles) followed by Exonuclease I treatment and MinElute clean up



2) QC after AMpure clean up to remove adaptor dimer



Pooled average fragment length = 895bp

<LibraryQC2> Check the DNA concentration using KAPA library quantification kit
The concentration of final library is 14.34 nM in 20 ul final volume (4-plex pool).

K. TROUBLESHOOTING

Step	Issue	Recommendations
Step 7	Ss cDNA concentration is lower than ~10 ng	• Ensure input total RNA is 5µg, and is of high quality. • Ensure complete removal of ethanol from the sample during cleaning steps prior to enrichment with streptavidin beads by removing all ethanol carefully from tubes/wells and incubating the eluate after collection. The presence of even small amounts of ethanol can interfere with the cap-trapping reaction and lead to poor cDNA recovery. • Ensure reactions are performed in the dark where specified. The oxidation and biotinylation reactions are light-sensitive, and cap-trapping will be less efficient when reagents are degraded by light. • Ensure as much eluate as possible is retrieved from the bead pellet during washing steps. Leaving excess volume in the tubes/wells will reduce cDNA yield. • Use streptavidin beads within 1 hour of preparation. Leaving prepared beads for too long before use may result in impaired capture of biotinylated fragments. • Yield of cDNA measured during this step is somewhat sample-dependent. If all the above steps have been followed correctly and yield is still low, this may be appropriate for your sample. You can continue to library preparation, or repeat with >5µg starting input if desired.

	Δ Ct between ACTB and rRNA > 4	• Ensure all wash steps are followed and all buffer removed between washes during capture with streptavidin beads. Insufficient washing will result in retention of unwanted uncapped transcripts and increased contamination with rRNA transcripts. • If using different qPCR primers to those included in Section G: Equipment and Materials above, ensure accuracy and efficiency of primers by appropriate testing before performing this assay. • Δ Ct measured during this step is somewhat sample-dependent. If the above steps have been followed correctly and Δ Ct is still high, this may be appropriate for your sample. You can continue to library preparation if desired, although the percentage of ribosomal reads in your final sequencing data may be higher than expected.
Step 16	Strong peak around 130 bp	• Perform extra AMpure clean up to remove adaptor dimer.
	Number of required PCR cycles too high (>10) despite good cDNA yield	• Ensure adaptors have been prepared and stored in buffer containing 100mM NaCl to ensure duplex stability. • Ensure both adaptor and cDNA molecules have been separately denatured prior to ligation steps. • High numbers of PCR cycles may be unavoidable depending on your normalisation pooling strategy and discrepancy between cDNA yields, as only a fraction of the cDNA may be carried through to library preparation. Consider whether a different pooling strategy may be preferable.
	Libraries too short (<800bp)	• Limit the number of PCR cycles performed to only as many as required to generate enough product for sequencing. Overamplification can lead to smaller final library product. • Ensure RNA has RIN value =>7. Using degraded RNA as input will result in shorter library fragments. • Ensure all work is performed in an RNAse-free environment, with RNAse-free reagents and equipment. RNA degradation occurring during the experimental setup will result in shorter library fragments.
Sequencing	Low Pass Filter %	• Use as many different 5' adaptors as possible to increase base diversity during the initial cycles. Low base diversity over the crucial first cycles of sequencing will result in poor sequencing metrics. • Increase PhiX % spike-in to sequencing pool to increase base diversity.
Analysis	Low mapping rate of sequencing reads (<90%)	• Ensure use of correct reference assembly for your sample. • Ensure sequencing data is of sufficient quality and read length.
	Low proportion of reads mapping to promoter regions (<70%)	• Ensure accuracy of reference annotation. • Ensure all wash steps are followed and all buffer removed between washes during capture with streptavidin beads. Insufficient washing will result in retention of unwanted uncapped transcripts and increased contamination with rRNA transcripts.

L. SOP VALIDATION DETAILS

This SOP has been developed according to '5' end–centred expression profiling using cap-analysis gene expression and next-generation sequencing' published in Nature Protocols (see 'Reference Documents' below), as well as the user guide for the commercially-available CAGE kit from DNAFORM, with input from company founder Yujiro Takegami. Data produced using this method has been analysed by Dr. Quan Nguyen, an expert in CAGE analysis, and has been judged to be of good quality. Testing has been performed on 2 cell types, both of human origin.

M. WASTE MANAGEMENT AND DISPOSAL

Solid and low-volume liquid waste generated through performing this protocol are to be disposed of into clinical waste bins according to IMB waste management protocol. Sharps are to be disposed of into puncture-resistant clinical sharps bins. There are no special waste disposal requirements associated with this SOP.

N. DATA RECORDS MANAGEMENT

We recommend all samples are tracked through unique sample ID from initial receipt of RNA through library generation and pooling. Results of all QC experiments including qPCRs, Oligreen assays, and BioAnalyzer runs can be stored as original data files and experiment summaries as necessary, employing a sample tracking system that can easily link these documents back to each individual experiment..

Specifically:

For Oligreen assays, store a copy of the exported excel spreadsheet.

For qPCR for rRNA contamination, store a copy of the .eds file, and exported results file with ΔCt calculations.

For qPCR for library pooling before final PCR, store a copy of the .eds file, and completed CAGE sample pooling sheet.

For BioAnalyzer runs, store a copy of the .xad file and exported pdf file.

For qPCR for library quantification, store a copy of the .eds file, and completed KAPA Library Quant Data Analysis sheet.

After sequencing, fastq files can be transferred to the relevant RDM record for storage and dissemination.

O. REFERENCE DOCUMENTS

Risk assessments associated with this SOP are available in the IMB Risk Management Database in WebDB:

- Risk Assessment ID #2591 "Cap-analysis gene expression (CAGE) with CAGE Library preparation kit and in-house protocol". A pdf version of this risk assessment is available on the CAGE development page of the GIH website.

Other SOPs referenced in this SOP are available on the CAGE development page of the GIH website:

- SOP002-02 CAGE Quant-iT Oligreen ssDNA assay
- SOP003-02 CAGE rRNA contamination qPCR
- SOP004-02 CAGE KAPA library quant qPCR

The 'CAGE sample pooling template' spreadsheet is available on the CAGE development page of the GIH website.

The Nature Protocols paper referred to in this document and used as the basis for the development of this protocol is available at DOI: 10.1038/nprot.2012.005. (PMID: 22362160)

P. QUALITY CONTROL (QC) AND QUALITY ASSURANCE (QA) SECTION

The following checks can be used to assess the quality of the final library:

- ACTB – rRNA ΔCT at ssDNA QC check 1 – expected to be <4
- total quantity of ssDNA at ssDNA QC check 2 – expected to be 15-30ng
- fragment length of final library at Library QC check 1 – expected to be >800bp
- final library concentration at Library QC check 2 – expected to be 10-20 nM for 4-plex library

The following checks can be used to assess the quality of the final data:

- Mapping rate – proportion of sequencing reads mapping to the genome. >95% is good, >90% is acceptable.
- Read distribution – proportion of sequencing reads mapping to different regions of the genome. >80% mapping to the promoter region is good, and indicates good enrichment for 5' end of RNA molecules.
- Number of identified transcription start sites/sample – This metric is particularly sample-dependent, so should be compared with previous data obtained with the same or similar cell type.

Scripts to perform these checks and others have been developed by Dr Quan Nguyen using publicly-available analysis packages and are available through Dr Nguyen's CAGE analysis repository at https://github.com/BiomedicalMachineLearning/CAGE_Analysis.